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Repeatability and method-dependent variation of blood parameters in wild-caught Great Tits *Parus major*

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Abstract. When interpreting responses to experimental manipulations or particular environmental cues, it is necessary to have previous knowledge about the natural variation of the response traits. We studied how nine blood parameters, including four enzymatic activities, varied with time in wild Great Tits by assessing their repeatabilities over periods of 45 days, in the same season (Spring or Autumn/Winter), in the same year and over a four years period. The accuracy of the measurements of these blood parameters was also assessed. Measurement error reflected essentially sample and time of storage rather than assay effects. Hematocrit and haemoglobin had low repeatabilities within Spring, ranging from 0.26 to 0.31; Heterophil/Lymphocyte ratio (H/L), white blood cell count (WBC), total plasma cholinesterase and red blood cell glutathione peroxidase (GSH-Px) activities had moderate to high repeatabilities over periods of 45 days (repeatabilities ranged from 0.47 to 0.81 for GSH-Px and H/L, respectively), but also during longer periods such as during Spring (total plasma cholinesterase activity) and Autumn/Winter (WBC, H/L and GSH-Px). Of the blood parameters measured, total plasma cholinesterase, glutathione peroxidase and the haematological parameters WBC and H/L seem relatively constant and therefore reliable indicators of Great Tit's physiological condition within, at least, a 45 days time frame.

Key words: repeatability, health state, condition, blood profile, *Parus major*, accuracy, glutathione peroxidase, plasma cholinesterase

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INTRODUCTION

The health profile and consequent performance of individuals in a population exposed to a certain set of environmental conditions can be used as a bioindicator to assess the state of populations and ecosystems (Ruiz et al. 2002, Artacho et al. 2007). This may be particularly important as an early warning reflecting the presence of stressors arising from many factors such as resources availability, competition or pollution (Furness 1993).

The blood parameters most commonly associated with the health of individuals are serological and haematological values (Averbeck 1992, Ots & Hõrak 1996, Hõrak & Ots 1998, Hõrak et al. 1998, Møller et al. 1998, Ots et al. 1998, Dubiec & Cichoń 2001) and for more specific purposes, such as detecting biochemical responses to xenobiotics, enzymatic biomarkers (Gage 1967, Cornish 1971, Kappus 1987, Peakall & Fournier 1992, Goede & Wolterbeek 1994, Walker et al. 2006).

In order to detect anomalous effects of the presence of stressors (e. g. exposure to pollutants) on blood profiles of individuals, ecologists need to understand their natural variation, the fluctuation of each parameter around its own average, the range of values of the population as well as the accuracy of their measurements. This is also important for studies which aim to detect effects of any experimental manipulation on blood parameters, especially in field conditions.

One way to understand the temporal variation in blood parameters is to study their stability or consistence when an individual is captured and sampled more than once. We can thus assess if those traits are stable or have fluid properties

reflecting temporary values. This can be achieved by estimating the repeatability of traits (Harper 1994, Hõrak et al. 2002).

Repeatability is estimated from the ratio of within and between individuals variance. The within individuals variance is due to environmental causes, whereas the among individuals variance is partly environmental (environmental differences that persist across time) and partly genetic determined (Falconer & Mackay 1996). Repeatability is often calculated to make inferences about: a) the reliability of multiple measurements of the same trait taken from the same individual (e.g. tarsus length is considered the same trait, because, after completed development, it does not change along an individuals' life), b) their environmental variance — if a trait is expected to change through an individuals life due to external factors, c) the constancy of a trait during development — if a trait is repeatable during development (between chick and adult) it can be considered the same trait, and d) in the area of quantitative genetics, to have an insight on the heritability of traits (Falconer & Mackay 1996, Dohm 2002); this is because repeatability can set the upper limit for heritability, since highly repeatable traits have the potential to be genetically determined.

Few studies have estimated repeatabilities of physiological parameters in natural populations over large periods of time (but see Chappell et al. 1995, Potti & Merino 1997, Bech et al. 1999, Potti et al. 1999, Hõrak et al. 2002). These studies focused mainly on metabolic performance, reproductive effort and mass loss but Hõrak et al. (2002) studied traits related to condition and health. They found that apart from basal metabolic rate, haematological parameters, such as white blood cell counts, were significantly repeatable over periods of 4 months. However, they studied Greenfinches *Carduelis chloris* kept in captivity, in which experimental conditions are controlled and consequently the results are not easily extrapolated to natural conditions; controlled conditions might reduce environmental variance and thus influence repeatability estimates.

In this study, we were mostly interested in assessing if differences between repeated measurements of the blood traits from the same individual arose due to measurement error or due to changes in the traits between observations, either because they are influenced by environmental factors (plastic traits) or because they account for biologically different traits over time (with different biological meaning). Therefore, we studied the repeatability of blood traits of wild Great Tits captured more than once over different time frames (45 days, same season, same year and 4 years). We also assessed the measurement error associated with the sample collection and laboratory measurement procedures (assay and time of storage effects), by performing additional analyses on a blood sample from a feral Pigeon *Columba livia*. These results provide insight as how to interpret, what to be aware of, and what cautions to take when studying blood parameters.

We aimed to understand the variation of these traits in natural conditions, in order to use Great Tit populations as bioindicators of environmental quality. The biology of this species is well known (Perrins 1979, Gosler 1993), which makes it a suitable model to use as a bioindicator.

We expect repeatabilities to decrease the further apart recaptures were made, because this should increase the probability of modification of the blood profile of each individual in a population.

METHODS

General methods and laboratory procedures

The nine blood profile traits considered in this study were biochemical: 1) total plasma protein (protein), plasma cholinesterase activities: 2) total cholinesterase (total ChE), 3) butyrylcholinesterase (BuChE), 4) acetylcholinesterase (AChE), 5) red blood cell glutathione peroxidase activity (GSH-Px), and haematological: 6) hematocrit (HCT), 7) index of red blood cell haemoglobin (Hb), 8) white blood cell count (WBC) and 9) heterophil/lymphocyte ratio (H/L).

To assess the measurement error due to the collection of blood samples and laboratory procedures on red blood cell haemoglobin index, total plasma protein and enzymatic activity measurements, we used blood from a Pigeon, from which it was possible to take a larger sample volume, allowing us to perform multiple enzymatic assays on the same sample. As measurement error was assessed using a different species, with probably different physiology due to different metabolic rate and feeding behaviour, among other factors, extrapolations to Great Tit measurements have to be made carefully.

Data to calculate repeatabilities of blood parameters in adult Great Tits were collected during a 4 years period (2003–2006) in Choupal, a mixed

deciduous woodland in Central Portugal (40°13′N, 8°27′W). Some data from Urso (42°55′N, 7°W) and Quiaios (40°21′N, 8°85′W) pinewoods (Autumn 2004, Winter/Spring 2005 and Winter 2006) and from the mixed wetland-forest of Madriz (40°7′N, 8°38′W) (Spring 2006) were also used. Number of samples for each blood parameter ranged between 213 and 246 from 86 to 95 individuals recaptured over the 4 years period. Great Tits were captured using mist-nets in Autumn and Winter, and in Spring, they were trapped in the nest boxes when feeding the 6–11 days old nestlings. A blood sample (100–150 µl) was taken from the brachial vein into a heparinized capillary tube.

Total plasma protein (mg/ml) was measured using Bradford Protein Assay, based on the Bradford dye-binding procedure (Bradford 1976). Total cholinesterase activity ($\mu mol.min^{-1}.ml^{-1}$) was assayed according to the method of Ellman et al. (1961), by following the increase of yellow colour produced by the reaction of thiocholine with 5,5-dithio[bis-2-nitrobenzoic acid] (DTNB) at 405nm. To distinguish between butyrylcholinesterase and acetylcholinesterase activity we selectively inhibited butyrylcholinesterase activity using tetraisopropylpyrophosphoramide (iso-OMPA). For glutathione peroxidase measurements ($\mu mol.min^{-1}.g^{1}$ haemoglobin) we used the method of Paglia & Valentine (1967) modified by da Silva & dos Santos (1991) which consists in measuring the rate of GSSG formation at 340 nm, as NADPH is converted to $NADP^{+}$. Enzymatic activities and total protein were measured in a Sunrise Inst. Microplate reader.

Hematocrit (%) was assessed as the percentage length occupied by red blood cells in relation to the total blood volume collected into a heparinized hematocrit capillary tube centrifuged (1200 rpm) for 10 minutes. Index of red blood cell haemoglobin (Hb, g/L) stands for the haemoglobin content in the 4x diluted haemolysate of red blood cells, prepared for the measurement of glutathione peroxidase activity, according to Paglia & Valentine (1967), and was measured with the cyanmethaemoglobin method at a wavelength of 540 nm (van Kampen & Zijlstra 1961), using a commercial kit (BioSystems S.A.). Blood smears from each bird were air-dried and stained using May-Gründwalds-Giemsa procedure and scanned under 1000x magnification. White blood cell count (WBC) stands for the estimation of the number of white blood cells in approximately 10000 red blood cells. Heterophil/lymphocyte ratio (H/L) was measured on the basis of the examination of 50 white blood cells, once the repeatability of measurements on 50 and 100 white blood cells was 0.94 ± 0.01.

Measurement error associated with sample collection and laboratory procedures

Approximately 4.5 ml of blood from one Pigeon were collected into 8 aliquots using heparin as anticoagulant. The first aliquot (aliquot 1) was used to investigate the effects of freezing and time of storage of the blood on enzymatic activities, protein and Hb (Experiment 1). The second aliquot (aliquot 2) was used to investigate the variation in measurements due to the assay, i.e., repeating the assay with the same operator and under the same conditions (Experiment 2). The remaining 6 aliquots (aliquot 3 to 8) were used to assess the variation due to sample effects (Experiment 3).

The preparation of blood for storage included its centrifugation (at 1200 rpm for 10 min) in order to separate plasma (which is ready for storage after centrifugation) from the red blood cells. The red blood cells were washed using 9 g/L NaCl solution. Subsequently they were lysed using ultrapurified water in the proportion of 1:4 ml of red blood cells to ultrapurified water. The haemolysate was separated from cell membranes by a centrifugation at 15000 g during 30 min. For haemoglobin stabilization, an equal volume of double strength Drabkin solution was added to the haemolysate (Paglia & Valentine 1967). The haemolysate sample was ready for storage at -80°C.

Aliquot 1 was processed using the procedure described above and then divided into tubes to be assayed fresh and after 1, 8, 15, 30, 45, 60 and 75 days of freezing (each assay consisted of a trial with 6 replicates). Aliquot 2 was also processed in the same way and frozen to be assayed in 3 different trials, each one consisting of 6 replicates. The three trials were made sequentially in the same day. The remaining aliquots, 3 to 8, were processed and frozen, and subsequently assayed in 6 different trials (each with 6 replicates) ran on the same day. In some cases the volume was insufficient and the final replicate number obtained, instead of 102 (no. of expected replicates from the design), was 49 for protein, 42 for Hb, 90 for total ChE, 85 for BuChE and AChE and 99 for GSH-Px. The effect of assay on haemoglobin and protein was not studied but the coefficient of variation was calculated respectively from out of 3 assays with one replicate each and 2 assays with 3 replicates each, respectively.

Means, standard deviations and coefficients of variation of enzyme activities, protein and Hb were calculated for each experiment. Data were assessed for normality (K-S test) and homogeneity of variances (Levene test). In order to assess the effects of freezing and time of storage of the blood on the enzyme activities, protein and Hb, we compared the measurements before and after freezing using a One-way ANOVA, and fitted a linear regression of enzyme activities, protein and Hb on time of storage in days. The effects of assay on enzymatic activities and effects of sample on enzyme activities, protein and Hb were tested using one-way ANOVA or Kruskal-Wallis test when the assumption of homogeneity of variances was not fulfilled.

Repeatabilities of Great Tit traits between recaptures

Repeatabilities were calculated as intraclass correlation coefficients, i.e. the ratio of among-individual variance to the sum of both among-individual and within-individual variances, following Lessells & Boag (1987). Variance components were estimated by One-way ANOVA. When data did not have a normal distribution, assessed using K-S test, transformed data was used. H/L ratio and WBC were transformed in all models, [1/(H/L+1)] and arcsin (WBC), but normality was not attained for the 4 years data. LnHb and lnGSH-Px were used in the ANOVAs for the same year and four years data, respectively. LnAChE and lnBuChE were used in the ANOVAs for 45 days and 4 year data. Data was not normal, even after transformation, for AChE in Spring, same year and four years data, and for total ChE in same year and four years data. Repeatability standard errors were calculated according to Becker (1984). We calculated repeatabilities of blood parameters in individual Great Tits recaptured over gradually larger periods of time: 45 days, same season (Spring: from beginning of April until the first week of July, Autumn: October/November or Winter: from mid-February until mid March), same year (2003/2004/2005/2006) and 4 years period (all 4 years data set). This allowed us to understand the degree of consistency of these parameters, how long they were stable and which seasons, and consequently life cycle periods, would have the greater influences on their values.

For the calculation of repeatabilities of blood parameters within the same season, data from within Spring recaptures were used separately from data of within Autumn or Winter recaptures. This is because in Spring, recaptures account for first and second broods, which might influence the physiological state of individuals.

We also fitted a linear regression of absolute differences in trait values on time-differences between recaptures, to determine whether paired observations became increasingly different with time.

Means, standard errors and coefficients of variation were calculated for each blood parameter using the four years data.

RESULTS

Measurement error associated with sample collection and laboratory procedures

The coefficients of variation of all experiments were relatively low and ranged from 3% (Hb) to 22% (protein; Table 1). There were significant

Table 1. Means, standard deviations (SD), sample size (N) and coefficients of variation (CV) of Pigeon blood parameters for each of the experiments (effect of time of storage, effect of assay, effect of sample). For definition of parameters see Methods. [(a)] — means of replicates of blood frozen for 1, 8, 15, 30, 45, 60 and 75 days.

Blood parameter	Effect of time of storage (Exp.1)			Effect of assay (Exp.2)			Effect of sample (Exp. 3)		
	Mean ± SD[(a)]	N	CV (%)	Mean ± SD	N	CV (%)	Mean ± SD	N	CV (%)
Protein (mg/ml)	47.1 ± 7.52	30	16	49.2 ± 3.46	8	7	108.3 ± 23.56	17	22
Total ChE ($\mu mol.min^{-1}.ml^{-1}$)	0.46 ± 0.021	42	5	0.47 ± 0.016	14	4	0.46 ± 0.020	28	4
AChE ($\mu mol.min^{-1}.ml^{-1}$)	0.021 ± 0.004	36	17	0.018 ± 0.003	14	18	0.021 ± 0.004	28	19
BuChE ($\mu mol.min^{-1}.ml^{-1}$)	0.44 ± 0.023	36	5	0.45 ± 0.016	14	4	0.44 ± 0.021	28	5
GSH-Px ($\mu mol.min^{-1}.g^{-1}hb$)	10.2 ± 1.56	42	15	11.6 ± 1.55	18	13	11.8 ± 1.05	33	9
Hb index (g/L)	48.7 ± 3.43	21	7	38.7 ± 5.27	3	13	30.6 ± 3.00	18	10

differences between fresh and frozen blood of the Pigeon in two of the cholinesterase enzyme activities: total ChE ($F_{1,10}$ = 9.71, p = 0.011) and BuChE ($F_{1,10}$ = 11.43, p = 0.007) but not in AChE. Plasma protein also differed between fresh and frozen samples ($F_{1,10}$ = 22.0, p = 0.003) but haemoglobin did not.

There were significant effects of time of storage on all enzyme activities and protein which explained from 18% (AChE) to 74% (protein) of their variation. The effect of enzymatic assay was significant only for AChE and it explained 55.4% of its variance. On the other hand, sample effects significantly influenced haemoglobin, total ChE, AChE and GSH-Px (Table 2).

Therefore, the effect of time of storage should be taken into account when measuring protein, plasma cholinesterases and glutathione peroxidase activities. Enzymatic assays used in this study were reliable and, although the assay had an effect on AChE measurements, the CV was low. Effect of sample should not be disregarded, especially for haemoglobin, total ChE, AChE and GSH-Px.

Repeatabilities of Great Tit traits between recaptures

The coefficient of variation of Great Tit blood parameters was relatively small, except for BuChE (46%) and haematological parameters (87 and 141% for WBC and H/L respectively; Table 3). Repeatabilities of blood parameters are presented in Table 4. Leucocyte related parameters had moderate to high repeatabilities over periods of 45 days (0.61 ± 0.13 and 0.81 ± 0.08 for WBC and H/L, respectively) and total ChE and GSH-Px had moderate repeatabilities (0.57 ± 0.16 and 0.47 ± 0.18, respectively) within the same period (Table 4). As the time period between recaptures increased (i. e. recaptures within the same season) the repeatabilities of WBC, H/L and GSH-Px remained moderate to high if recaptures were made in Autumn or Winter. In Spring, the repeatabilities of WBC and H/L decreased. If recaptures were in different seasons, repeatabilities decreased even further. Repeatabilities of HCT and Hb were low in Spring, whereas protein was moderately repeatable. Protein repeatability decreased within the same year and over the four years. BuChE repeatability was moderate in Spring but lower within the same year and four years period. Leucocyte related parameters seem to have relatively high repeatabilities, even over large periods of time such as one year.

Table 2. Effects of time of storage, enzymatic assay and sample on Pigeon blood parameters. For definition of parameters see methods and for units see Table 1. [a] - Equation from a linear regression of blood parameter on time of storage in days; F, p and R^2 from a One-way ANOVA; H from Kruskal- Wallis test.

Parameter	Effect of time of storage (Exp. 1)					Effect of assay (Exp. 2)				Effect of sample (Exp. 3)		
	Equation [a]	F	df	p	R^2	F	df	p	R^2	Statistic	p	R^2
Protein	protein = -0.250 time + 55.78	78.96	1.28	< 0.001	0.738	-	-	-	-	$H_{5,17}$ = 7.51	0.185	-
Total ChE	Total ChE = -0.00040 time + 0.471	10.67	1.40	0.002	0.211	3.04	2.11	0.089	0.356	$F_{5,22}$ = 3.84	0.012	0.466
BuChE	BuChE = -0.00048 time + 0.451	12.29	1.35	0.001	0.260	3.37	2.11	0.072	0.380	$H_{5,28}$ = 10.63	0.059	-
AChE	AChE = 0.000062 time + 0.019	7.68	1.35	0.009	0.180	6.84	2.11	0.012	0.554	$F_{5,22}$ = 5.32	0.002	0.547
GSH-Px	GSH-Px = -0.041 time + 11.58	29.44	1.40	< 0.001	0.424	0.64	2.15	0.539	0.079	$F_{5,27}$ = 3.52	0.014	0.395
Hb index	Hb = -0.014 time + 49.29	0.24	1.19	0.627	0.127	-	-	-	-	$F_{5,12}$ = 10.69	< 0.001	0.817

Table 3. Means, standard deviations (SD) and coefficients of variation (CV) of blood parameters in Great Tits recaptured during a 4 years period. For definition of parameters see methods. Sample sizes varied between 213 and 246.

Blood Parameter	Mean ± SD	CV (%)
Protein (mg/ml)	65.31 ± 13.29	20
Total ChE (μmol.min^{-1}.ml^{-1})	0.349 ± 0.039	11
BuChE (μmol.min^{-1}.ml^{-1})	0.079 ± 0.036	46
AChE (μmol.min^{-1}.ml^{-1})	0.273 ± 0.040	15
GSH-Px (μmol.min^{-1}.g^{-1}hb)	10.73 ± 2.17	20
HCT (%)	53.60 ± 4.32	8
Hb index (g/L)	30.51 ± 6.56	22
WBC	11.95 ± 10.38	87
H/L	0.81 ± 1.15	141

From the regressions of differences in trait values on differences in time period between recaptures, we had no evidences of larger differences of paired observations when they were sampled further apart. In fact, most of the regressions were non-significant, except for Hb (Hb_{obs2} - Hb_{obs1} = 4.61 + 0.037*$date_2$ - $date_1$, $F_{1,60}$ = 5.76, p = 0.020, R^2 = 0.088), total ChE (total ChE_{obs2} - totalChE_{obs1} = 0.023 +0.00019* $date_2$-$date_1$, $F_{1,56}$ = 4.05, p = 0.049, R^2 = 0.067) and H/L (H/L_{obs2} - H/L_{obs1} = 1.33-0.003*$date_2$-$date_1$, $F_{1,66}$ = 8.13, p = 0.006, R^2 = 0.11), but the variance explained by the models was very small.

DISCUSSION

Measurement error associated with laboratory procedures was found to arise essentially from sample effects and time of storage, and to a less extent from assay effects, which only influenced AChE measurement.

In Great Tits, WBC and H/L were moderately to highly repeatable over periods of 45 days and even if individuals were recaptured within the same season (Autumn or Winter). Total ChE was moderately repeatable within Spring and GSH-Px was moderately repeatable within periods of 45 days and within Autumn and Winter. When recaptures were made further apart, repeatabilities tended to decrease.

Measurement error associated with sample collection and laboratory procedures

There were significant differences between plasma cholinesterase activities (except AChE) and protein measurements using fresh and frozen blood and it was also found that the duration of storage affected all enzymatic activities and protein of the Pigeon blood. Hill & Murray (1987)

Table 4. Repeatabilities (r) and corresponding standard errors (SE) of blood parameters of Great Tit individuals recaptured over time periods of: 45 days, same season (Spring - average interval = 46.5 days and Autumn/Winter - average interval = 18.7 days), same year and 4 years. ns — number of samples; ni — number of individuals; p from a One-way ANOVA with ring number as a fixed factor. For definition of parameters see methods.

Blood	45 days			Same season (same year) - Spring			Same season (same year) - Autumn/ Winter			Same year			4 years period		
Parameter	ns/ni	r ± SE	p	ns/ni	r ± SE	p	ns/ni	r ± SE	p	ns/ni	r ± SE	p	ns/ni	r ± SE	p
Protein	44/22	0.196 ± 0.21	0.180	76/38	0.445 ± 0.13	0.002	19/9	0.018 ± 0.31	0.47	134/62	0.330 ± 0.11	0.002	246/95	0.190 ± 0.08	0.005
Total ChE	38/19	0.568 ± 0.16	0.004	70/35	0.393 ± 0.14	0.008	17/8	-0.021 ± 0.33	0.51	125/58	0.124 ± 0.12	0.15	236/91	-0.013 ± 0.07	0.57
BuChE	34/17	-0.178 ± 0.24	0.762	60/30	0.354 ± 0.16	0.024	17/8	-0.602 ± 0.18	0.97	113/52	0.248 ± 0.12	0.022	219/86	0.122 ± 0.08	0.059
AChE	36/18	-0.217 ± 0.22	0.816	62/31	-0.130 ± 0.18	0.76	17/8	-0.062 ± 0.33	0.56	111/51	0.003 ± 0.12	0.49	221/87	0.234 ± 0.08	0.001
GSH-Px	40/20	0.468 ± 0.17	0.015	66/33	-0.161 ± 0.17	0.82	19/9	0.557 ± 0.22	0.03	123/56	0.135 ± 0.12	0.12	230/89	0.085 ± 0.08	0.13
HCT	44/22	0.058 ± 0.21	0.395	76/38	0.315 ± 0.15	0.024	17/8	-0.072 ± 0.36	0.57	135/62	0.244 ± 0.11	0.015	242/93	0.102 ± 0.08	0.080
Hb index	42/21	-0.107 ± 0.22	0.683	74/37	0.263 ± 0.15	0.054	19/9	-0.302 ± 0.27	0.82	135/62	0.039 ± 0.11	0.36	243/93	0.008 ± 0.07	0.45
WBC	46/23	0.606 ± 0.13	<0.001	80/40	0.506 ± 0.12	<0.001	17/8	0.822 ± 0.11	<0.001	138/65	0.492 ± 0.09	<0.0001	239/94	0.257 ± 0.08	<0.001
H/L	42/21	0.808 ± 0.08	<0.0001	70/35	0.517 ± 0.12	<0.001	15/7	0.743 ± 0.16	0.007	121/58	0.449 ± 0.10	<0.0001	213/87	0.249 ± 0.08	0.001

compared cholinesterase activities between fresh and frozen plasma, in four passerine species, and found significant differences only for the European Starling *Sturnus vulgaris*. In the Japanese Quail *Coturnix japonica* there were differences in plasma cholinesterase activity between fresh and frozen blood only for females, with an increase in plasma ChE of 19% after 29 days of freezing, comparing to fresh samples. They explained that their findings could be due to dissociation of any constituent of female blood from the enzyme during freezing (Hill 1989). Also, when these enzymes are inhibited by organophosphates, changes in enzyme-inhibitor complexes, caused by freezing, may influence results of cholinesterase analysis (Westlake et al. 1985). In our study, there was an increase in AChE activity with time of freezing but not in total ChE or BuChE, whose activities decreased. On the other hand, Fairbrother et al. (1991) found total cholinesterase enzyme activity to be stable up to 136 days in samples stored at -70°C. Error introduced by freezing and time of storage should be taken into account when studying plasma protein and enzyme activities (plasma cholinesterases and red blood cell glutathione peroxidase) but once our Great Tit samples were all assayed after freezing, due to time limitations, and were kept in the freezer for the maximum period of 45 days, we are confident that the act of freezing and time of storage has not introduced large variation among our Great Tit measurements, allowing comparisons among them.

There were no significant differences between assays of the same sample, except for AChE. Incubation time of the reaction mixture with butyrylcholinesterase inhibitor might have been insufficient, leading to erroneous AChE measurements (Fairbrother et al. 1991). The associated coefficient of variation was relatively small (18%), so we presume that measurement error related with assay of enzyme activities was minor. The method according to which we measured cholinesterase activity, originally described by Ellman et al. (1961), produced a maximum CV of approximately 4.9% for different assays of bovine red blood cells cholinesterases, which is similar to the value obtained in our study for total ChE and BuChE (for which assay did not introduce significant differences).

Concerning the effects of sampling, it might be a problem as it affected all enzyme activities, except BuChE, and Hb measurements. However, the coefficients of variation were rather small. Previous studies have indicated that CV in cholinesterases activity measurements is in the region of 4% using duplicate samples of blood taken at the same time (Kane 1958, Witter 1963). The fact that the samples taken from the Pigeon were much larger than those taken from the Great Tits makes extrapolations difficult. One way to reduce this problem is probably to take an amount of blood the most similar possible between samples, once the relationship between the amount of heparin and blood influence the clotting of the sample, possibly its turbidity and consequently the spectrophotometric determinations. Haemolysis of red blood cells prior to centrifugation can also alter the plasma sample due to contamination with red blood cells content, and influence measurements of light absorbance (Fairbrother et al. 1991). Presence of lipids was referred to influence cholinesterase activity measurements due to interference with the passage of light through the sample (Bowers et al. 1975). Sampling similar amounts of blood in different Great Tit individuals might be difficult because of weather conditions, clotting time for each bird, etc. We noticed that the amount of clotting differed between Pigeon samples, especially between aliquots used for the third experiment (3 to 8).

It should be taken into account that results obtained from the 3 different experiments cannot be compared because analyses were made with different times of storage for the 3 experiments, and sample volumes differed among the aliquots.

The fact that the CV related with sample effects for protein was similar to the CV obtained for Great Tit populations suggests that the protein measurements are probably not accurate and that all population variation found in Great Tits could be due to sample effects. However, in spite of these results having the potential to provide an estimation of how large the measurement error can be, when measuring these blood parameters in birds such as Great Tits, these extrapolations must be made cautiously. We found that total cholinesterase activity in Pigeon plasma was mainly due to BuChE whereas in Great Tits it was mainly due to AChE (as in other passerines; M. J. Hooper — unpublished data). So, when extrapolating from Pigeon to Great Tit results, the measurement error might be overestimated for AChE and underestimated for BuChE.

Repeatabilities of Great Tit blood parameters between recaptures

The high coefficients of variation of haematological parameters and BuChE in Great Tit

populations indicate high inter-individual variability. Ots et al. (1998) had already reported that the H/L ratio revealed a great variation at the population level with a high CV, which is in accordance with our results. The coefficient of variation of BuChE obtained in this study was high compared with that obtained in other studies. Hill & Murray (1987) reported a CV lower than 25% within the same seasonal sampling period for captive birds; also Gage (1967) mentioned a CV for plasma total cholinesterases of 15–25% in populations not exposed to pollutants.

Hematocrit and haemoglobin seem to have low repeatabilities, at least during long periods of time, such as one year or even season. We suppose that this finding is due to variation in these plastic and predominantly environmental determined traits. This was expected as these traits are known to respond to environmental changes such as food availability and nutrition (Rattner et al. 1987, Merino & Potti 1998, Totzke et al. 1999), energy expenditure (Swanson 1990, Kostelecka-Myrcha et al. 1993, Saino et al. 1997a,b) and seasonally, due to light (Riddle & Braucher 1934), endocrine factors (Riddle & Braucher 1934, Kern et al. 1972) and temperature (de Graw et al. 1979). For a detailed review of sources of variation of hematocrit in birds see Fair et al. (2007). We also expected protein to have low repeatabilities, but that was not the case, as repeatability was moderate in Spring and throughout the year. This indicates that measurement error associated with sample effects on protein was relatively small for Great Tits, contrary to our first assumption (see Discussion). Protein repeatability might be higher in periods of large and constant food availability, such as Spring, as protein is an indicator of nutritional status and short-term condition, reflecting what the bird ate recently and what has been absorbed and mobilized to body tissues (Lewandowski et al. 1986, Brown 1996). Hõrak et al. (2002) found that protein concentration was significantly repeatable during 4 and 8 days but not during 4 months, even in more controlled conditions than those used in this study, i.e. Greenfinches kept in captivity.

On the other hand, haematological parameters such as WBC and H/L had high repeatabilities, especially in Autumn/Winter and over periods of 45 days, suggesting that they are reliable indicators of the individuals' condition during those periods. WBC and H/L are related with immune function and involved in the process of infection and inflammation (Campbell 1995). Because repeatability may set the upper limit to heritability (Falconer & Mackay 1996), the results obtained in this study are in accordance with the finding of a heritable component of variation in immune response traits (Brinkhof et al. 1999, Gleeson et al. 2005, Kilpimaa et al. 2005, Cichoń et al. 2006, but see Christe et al. 2000). Hõrak et al. (2002) also found WBC and H/L to be consistent during a four months period, between Autumn and Spring ($r = 0.88$ and 0.54, respectively), which they explained as interindividual differences in the prevalence of chronic infections. In Spring, the repeatabilities of WBC and H/L were lower. This suggests that Spring is a period with strong effects on leucocyte related parameters, probably related with the fact that recaptures reflect first and second clutches. These effects may be caused by the high energetic demands of breeding activity and consequent stressful conditions the breeding pairs have to face (Drent & Daan 1980, Merilä & Wiggins 1997), which are known to affect H/L, a stress indicator in birds (Gross & Siegel 1983, Maxwell 1993, Hõrak et al. 1998, Vleck et al. 2000). Higher parasite transmission rates and higher susceptibility to parasites, due to immunossupression related with allocation of resources for breeding activity (Nordling et al. 1998, Christe et al. 2002) in Spring, could affect WBC and H/L and decrease their repeatabilities.

With respect to enzymatic activities, glutathione peroxidase was moderately repeatable over periods of 45 days and within Autumn and Winter recaptures. GSH-Px is an antioxidant enzyme (Ames et al. 1993). During Autumn and Winter, processes related with high energy expenditure (e.g. reproductive activity) and inflammation, and which originate reactive oxygen species (Ames et al. 1993, Alonso-Alvarez et al. 2004), may not occur or are less common, and, therefore, variation on the levels of oxidative stress might be reduced during those seasons. The CV of GSH-Px obtained by Paglia & Valentine (1967) in assays of repeated human samples, obtained within a period of 2 months was 14.3%. Faraji et al. (1987) found a CV of 8.1% along a period of 17 days for this enzyme in human blood. Cholinesterases were moderately repeatable over 45 days (total ChE) and in Spring (total ChE and BuChE). Repeatability of total ChE decreased from recaptures made within 45 days to recaptures made within Spring. This could be related with hormone effects on cholinesterase activities. Rattner & Fairbrother (1991) reported that serum cholinesterase activities depended on the

reproductive status of the birds. Serum cholinesterase was reported to be very stable in healthy human individuals not exposed to pollutants, with a CV of 8.4% in 82 individuals tested 373 times over a period of 1–250 weeks (Wetstone & LaMotta 1965), and variation between individuals was greater than within individuals (Sawitsky et al. 1948). The fact that both total cholinesterase and GSH-Px varied little during periods of 45 days (or even longer as one season), suggests that they are reliable indicators of an individuals' health, as they seem to reflect relatively long-term components of an individuals physiology, than other studied parameters (with exception of WBC and H/L).

We conclude that of all blood parameters measured, haematological parameters (WBC and H/L), total cholinesterases and GSH-Px seem the most reliable indicators of the health of the Great Tits, at least during periods of 45 days, when they are relatively stable.

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STRESZCZENIE

[Powtarzalność parametrów biochemicznych i hematologicznych krwi oraz źródła ich zmienności u bogatek]

Podczas badań prowadzonych na bogatkach badano w jaki sposób zmienia się w czasie 9 parametrów krwi — poziom cholinesterazy plazmatycznej, całkowity poziom cholinesterazy (ChE), butyrylocholinesterazy (BuChE), acetylocholinesterazy (AChE), peroksydazy czerwonych krwinek (GSH-Px) a także hematokryt (HCT), poziom hemoglobiny w czerwonych krwinkach (Hb), liczba białych krwinek (WBC) i stosunek heterofilów/leukocytów (H/L). Materiał pozyskiwano od bogatek podczas czterech lat badań (2003–2006) w Choupal, w środkowej Portugalii oraz kilku innych miejscach. W sumie zebrano od 213 do 246 próbek, przy czym 86–95 dla tych samych osobników chwytanych ponownie. Ptaki były chwytane w sieci (jesienią i zimą) lub w skrzynkach podczas karmienia piskląt (w wieku 6–11 dni). Próbki krwi (100–150 µl) pobierano z żyły skrzydłowej do kapilar zawierających heparynę. Przed rozpoczęciem badań sprawdzono także błąd pomiaru wynikający z zastosowanych procedur laboratoryjnych — używając krwi gołębia miejskiego (Tab. 1, 2).

Okazało się, że u bogatek powtarzalność wyników u tych samych osobników chwytanych ponownie była znaczna, z wyjątkiem BuChE (zmienność 46%) oraz WBC (87%) i H/L (141%) (Tab. 3, Tab. 4). Wraz z wydłużeniem okresu między kolejnymi schwytaniami tego samego osobnika w danym sezonie powtarzalność wyników dla WBC, H/L oraz GSH-Px pozostawała średnia do wysokiej, jeśli powtórne odłowienie miało miejsce jesienią lub zimą. Wiosną powtarzalność dla WBC i H/L spadała. Gdy powtórne schwytania miały miejsce w różnych latach, powtarzalność pomiarów była jeszcze mniejsza. Dla HCT i Hb powtarzalność była niska na wiosnę, natomiast w przypadku białka utrzymywała się na średnim poziomie lecz spadała później w sezonie i w kolejnych latach. Podobne wyniki uzyskano też dla BuChE. Parametry związane z analizą leukocytów wykazywały znaczne podobieństwo, nawet w okresie jednego roku.

Badania wskazują, że najbardziej wiarygodnymi wskaźnikami kondycji u bogatek są parametry hematologiczne (WBC oraz H/L) a także CHE i GSH-Px, które są relatywnie stabilne.